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Characterisation and Identification of lactic acid bacteria isolated from Moroccan raw cow's milk

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Abstract

Lactic acid bacteria have significant potential for use in food processing and especially dairy products because they are safe and naturally dominate the microflora of many foods during storage.This study was to characterise and select 280 isolates from raw cow milk in area of Oujda of lactic acid bacteria such as Streptococcus; Lactococcus, Lactobacillus and Enterococcus from cow's raw milk samples collected in area of Oujda .According to the morphological, physiological, and biochemical tests,12 of Lactic Acid Bacteria were identified at species level: *Streptococcus salivarius subsp. Thermophilus*; *Streptococcus equinus, Lactococcus lactis subspcremoris*; *Leuconostocmesenteroides subsp. Cremoris; Pediococcusdamnosus: Lactobacillus delbrueckii subsp. Bulgaricus*; *Lactobacillus plantarum; Lactobacillus delbrueckii subsp. Lactis, Lactobacillus brevis; Lactobacillus delbrueckiisubsp.delbrueckii.*In view of the results generated, bacterial species isolated have in great demand in industrial scale for its acidifying and flavoring abilities, like Streptococcus salivarius subsp. Thermophilus, which would be interesting for a future study have used it as an adequate starter culture for the manufacture of dairy products.

1. Introduction

Milk and dairy product is nutritious food items containing numerous essential nutrients and characterised by a rich biodiversity of lactic acid bacteria.

Raw milk is the most used product for obtaining useful cultures for food industry. Endeed, this milk's lactic acid bacteria plays an important role in the development of organoleptic characteristics (flavors and nutritional qualities) of fermented dairy products like fermented milk and cheese[1, 2].

Thereby, in the manufacture of various fermented dairy products, the selection of lactic acid bacteria is based on the production of lactic acid, peptides and aromatic compounds[3-5]. These original lactic flora of raw milk has been the subject of several research studies[6-11].

Lactic acid bacteria (LAB) represent an heterogeneous group of microorganisms characterised by the production of lactic acid as the main metabolic end product from glucose and reducing pH. The acid produced promotethe growth conditions of a wide variety of pathogens and spoilage microbes.Whereas, LAB are more tolerant to lower pH environments[12]. They are gram positive, non sporing, catalase negative, aerotolerant, acid tolerant, and a strictly fermentative[11].

The identification tests have allowed a classification of lactic acid bacteria in groups : Streptococcus, Lactococcus, Enterococcus, Leuconostoc, Lactobacillus and Pediococcus[10, 13].The identification of LAB is

mainly based on morphology, carbohydrates fermentation, production of CO2, capacity to grow at high salt concentrations, and growth at different temperatures[4, 14].
The aim of this study is the characterisation and identification of the LAB isolated from raw cow milk in order to select strains for possible uses in the manufacture of dairy products[6, 7].

2. Material and Methods

2.1. Sampling

Samples of raw milk were collected from dairy farms and randomly selected from area of Oujda. A total of 30 milk samples were immediately cooled and brought to the laboratory in an icebox and stored at -20°C until analysed.

2.2. Isolation of LAB

Ten mL of raw milk from each sample were homogenised with 90 mL of sterile tryptone salt broth. The stock solution obtained (10^{-1}) was used for making suitable serial dilutions up to 10^{-8} by incorporating 1 mL into 9 mL of the diluent [9]. a quantity of 1 mL is taken from each dilution of the sample to be analysed to drop in some boxes of Petri sterile. Isolation of Lactococcus was done using M17 (Biokar)[15] and MRS media (Biokar)[16] for lactobacilli, after incubation at 42°C for 24-48h and 37 °C for 48-72 h respectively[17]. All strains were randomly picked from plates for the Macroscopic examination. The selected colonies were purified by repeated streaking on the appropriate agar media[18, 19].
The pure isolates of lactic acid bacteria were kept at 4°C and revived every 4 weeks in order to preserve them [7, 20].

2.3. Phenotypic characterisation

The isolated LAB were first enriched using 4 mL of broth MRS and M17 medium and incubated for 24 h at 30°C and 42°C respectively[21]. Phenotypic characterisation of lactic acid bacteria strains was based on cell shape, Gram stain, catalase production.

2.3.1. Macroscopic analyses

The macroscopic characters of colonies of lactic acid bacteria is studied with a binocular loupe (Motic) in order to describe their morphology; color, surface, elevation, bord, aspect and opacity.

2.3.2.Microscopic analyses

The microscopic observation was made using a microscope (Motic) linked to a computer and a camera to take pictures with the objective 100.

2.4. Physiological test

The isolates were characterised based on Gram staining and catalase test. Only Gram-positive and catalase negative strains were retained. Further identification was performed by using the ability to grow at different temperatures 10°C 15°C and 45°C; catalase assay tolerance to 6.5% NaCl, hydrolysis esculin, Gas production, hemolysis test.

2.4.1. Growth at different temperatures

The growth test at different temperatures consists of inoculating the young cultures of the lactic acid bacteria isolates in tubes containing the broth MRS and M17 medium, then incubated at 10°C 15°C, and 45°C for 72h. The growth of lactic acid bacteria indicates the tolerance of this temperatures.

2.4.2.Catalase assay

The catalase activity was determined by adding of hydrogen peroxide (H_2O_2) 3%(v/v) (sigma) onto the cultured colonies, according to [22].

2.4.3.Sensitivity to salt

The isolates were tested for tolerance to 6.5% (w/v) NaCl concentrations in MRS and M17 broth medium. The tubes were inoculated with young cultures and then incubated at 37°C and 42°C /48h [23].

2.4.4.Esculin hydrolysis

This hydrolysis is evidenced by the blackening of the medium agar esculin. The test is read after 24h to 48h at 37°C compared to negative control.

2.4.5.Gas production

The homo or hetero fermentative character of the isolates is studied basing their ability or not to produce gas (CO_2). The test was studied using inverted Durham tubeson MRS and M17 broth medium[24, 25].

2.4.6. Haemolysis test

For haemolysis test, the LAB isolates were cultured using MRS and M17 broth at 37°C for 15 h and then transferred onto blood agar (biokar) supplemented with 5% sheep's blood. The inoculated plates were incubated at 37°C for 24 h [26, 27]. the haemolytic reaction was evaluated by observing both the partial hydrolysis of red blood cells and the production of a green zone (α-hemolysis), as well as the total hydrolysis of red blood cells producing a clear zone around bacterial colony (β-hemolysis) or no reaction (γ-hemolysis).

2.5. Biochemical Identification

The biochemical identification and Carbohydrate fermentation of Lactococcus were carried out on each isolate using Api 20 STREP (Bio Mérieux, France) according to the manufacturer's instructions[28]: acetoïn production; hyppurate, esculin and arginine hydrolysis; pyrrolidonyl-aralamydase, α-galactosidase,β-galactosidase, β-glucuronidase activity and utilisation of ribose, arabinose, mannitol, sorbitol, lactose, trehalose, inulin, raffinose, starch, glycogen and glycerol. Apistreeps were incubated at 36°C ± 2°C and examined after 4 and 24 hours.

In other hand, Identification of lactobacillus were accomplished by using API 50 CHL micro-identification systems (Bio Mérieux, France) and incubated at 36 at 36°C ± 2°C for 24 and 48 hours.

The interpretation of the fermentation profiles was facilitated by the use of the computer-aided database ''APIWEB'' (Bio Mérieux).

3. Results and discussion

3.1. Macroscopic and Microscopic morphology

A total of 280 strains obtained from 30 samples of raw cow milk from area of Oujda. The general properties (shape; border; rounded; convex; surface, smooth; and colour: white) of all strains allowed us to observe a big white lenticular colonies, cocci, diplococcic in short chains (Enterococci, 120 strains, 42.86%). White lenticular and convex colonies (Figure1), cocci, diplococci in chains (Figure2) (Streptococci, 90 strains, 32.14%). White small round colonies with a white halo, long rods, alone or in chains (Lactobacilli, 70 strains, 25%) (table 1) [8, 10, 29]. Out of the 280 isolates of the LAB, 12 isolates were identified at species level. According to the morphological, physiological, and biochemical tests.

3.2. Physiological test

Microscopic observation showed that all strains of LAB are Gram positive. All LAB strains (1, 2, 3, 4, 5, 6, 7, 8, 9, 11, 12) are Gram-positive and catalase negative which is correlated with the previous results [7]. The absence

of the catalase enzyme in LAB which disintegrates H_2O_2 results in the accumulation of H_2O_2 in the fermented food medium[30].

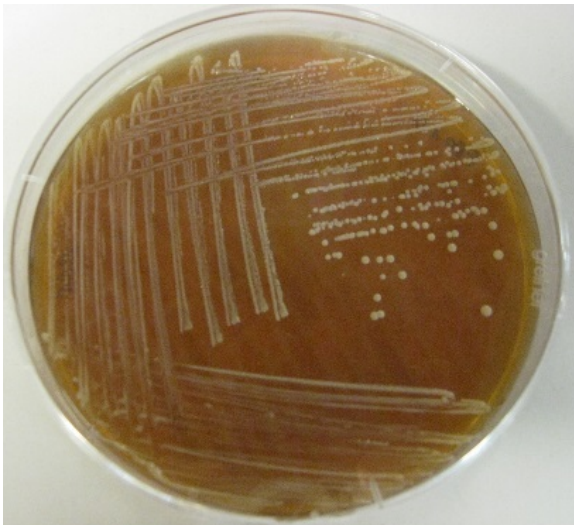

Figure*1*: Microscopic view of streptococci

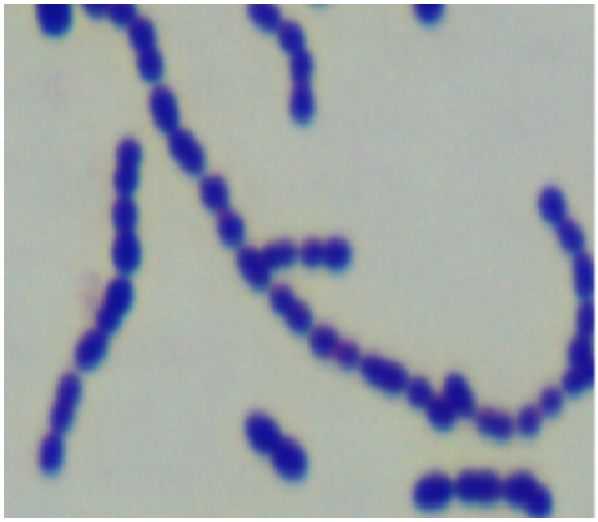

Figure2 : Microscopic view of streptococci

The selected LAB showed a good growth towards different temperatures, 58.33% of LAB (1, 2, 6, 7, 9, 11) grew at 10°C (Table 1), 66.66% (1, 2, 4, 5, 6, 7, 9, 11) grew at 15°C and 41.66% (3, 4, 8, 10, 11) grew at 45°C. This result is in agreement with that found by Badis, Guetarni [31]. LAB can generally be classified in terms of optimum growth temperatures, namely mesophilic and thermophilic LAB.

For sensitivity to salt all strains (99%) of LAB strains (1, 2, 3, 5, 6 7, 8, 9, 11, 12) don't growth at 6.5% of NaCl unlike (LAB 4) which is correlated with the previous results [25].

In addition to this, just 25% of LAB strains (4, 7, 9) produces the esculinase (β-Glucosidase) enzym. However, none of the strains produced haemolysin in sheep's blood agar except (LAB 4) that is variable between (α-hemolysis) and (β-hemolysis), this result is in agreement with that found byAbushelaibi, Al-Mahadin [32].

Finally, 83.33% of LAB strains (1, 2, 3, 4, 5, 7, 8, 9, 12) produce lactic acid using glucose without gas, unlike 16.66% (6; 11) that produce CO2 in the (M17, MRS) broth medium this result is in agreement with that found by Kacem, Zadi-Karam [33].

The production of lactic acid as the major product from the energy-yielding fermentation of sugars [34-36].

LAB also produces carbon dioxide (CO_2) as an end-product of glucose fermentation [30]. 12 isolates were then selected based on the above tests for further identifications by using API kits.

3.2. Biochemical identification

LAB isolated (12 strains) were identified at species levelswere investigated using API 50 CHL and API 20 Strep as *Lactococcus lactis subspcremoris* (LAB 1), and *Lc. lactis subsp. Lactis* (LAB 2), *Streptococcus salivarius subsp. Thermophilus* (LAB 3), *Enterococcus durans* (LAB 4), *St. equinus* (LAB 5) (Table 2). *Leuconostocmesenteroides subsp. Cremoris* (LAB 6), *Pediococcusdamnosus* (LAB 7), *Lactobacillus delbrueckii subsp. Bulgaricus* (LAB 8), *Lb. plantarum* (LAB 9), *Lb. delbrueckii subsp. Lactis* (LAB 10), *Lb. brevis* (LAB 11), *Lb. delbrueckii subsp. Delbrueckii* (LAB 12) (Table 3).

Lc. lactis subsp. cremoris and *Lc. lactis subsp. lactis* produce acetoïn (Table 2) who contribute to the flavour of the fermented product. According to the following research [37, 38], these species given to fermented milks a volatile aroma compounds during growth and exopolysaccharides which improve the texture, viscosity and consistency.

Table 1: Physiological characteristic of the isolates LAB

	Isolates of LAB											
Tests	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Forme	c	c	c	c	c	c	c	b	b	b	b	b
Grame	+	+	+	+	+	+	+	+	+	+	+	+
Catalase	-	-	-	-	-	-	-	-	-	-	-	-
Growth at different temperature												
(10°C)	+	+	-	+	-	+	+	-	+	-	+	-
(15°C)	+	+	-	+	+	+	+	-	+	-	+	-
(45°C)	-	-	+	+	NT	-	V	+	-	+	-	+
Growth in 6.5% NaCl	-	-	-	+	-	-	-	-	-	-	-	NT
Esculin	-	-	-	+	-	-	+	-	+	-	-	-
Hémolyse(β),	-	-	-	v	-	-	-	-	-	-	-	-
Hémolyse(α)	-	-	-	v	-	-	-	-	-	-	-	-
Fermentation type	homo	homo	homo	homo	homo	heter	homo	homo	homo	homo	heter	homo

NT: not tested+: Positive reaction: Negative reaction; v: variable; c: cocci; b: bacilli

St. salivarius subsp. Thermophilus were able to ferment lactose and produce acetoïn (Table 2). Is generally recognised as safe (GRAS) and found in dairy environments according to Delorme [39], De Vuyst and Tsakalidou [40], this strain is mixed with *Lactobacillus delbrueckii subsp. Bulgaricus* are used in various dairy fermentations, for example; yoghurt, fermented milk and types of cheeses[39, 41].

Enterococcus durans was homo fermentative and produced ammonia from hydrolysis of arginine, producing acetoïn and fermenting lactose (Table 2). Nevertheless, unlike most LAB genus, enterococci are not GRAS. This is due in their role pathogens. However, they are safe to use in dairy fermentations [42-45]including the production of antimicrobial bacteriocins and their role in the ripening of aromas, aromas and textures [43, 46-48].

Table 2: Identification of lactococci isolated from row cow milk by API 20 Strep

	Isolates of LAB				
	1	2	3	4	5
Acetoïn production	+	+	-	+	+
Hippurate hydrolysis	-	-	-	-	-
Esculin hydrolysis	-	+	-	+	+
Pyrrolidonylarylamidase	-	-	-	+	-
α-Galactosidase	-	-	+	-	-
β-Glucuronidase	-	-	-	-	-
β-Galactosidase	-	-	-	+	-
Alkaline phosphatase	-	-	-	-	-
Leucine amino peptidase	+	+	+	+	+
Arginine hydrolysis	-	+	-	+	-
Ribose	-	+	-	+	-
Arabinose	-	-	-	-	-
Mannitol	-	-	-	-	-
Sorbitol	-	-	-	-	-
Lactose	+	-	-	+	-
Trehalose	-	+	-	+	-
Inulin	-	-	-	-	-
Raffinose	-	-	-	-	-
Amidon	-	+	-	-	-
glycogen	-	-	-	-	-

+: Positive reaction: Negative reaction;
1 Lactococcus lactis subspcremoris, 2 Lactococcus lactis subsp. Lactis, 3 Streptococcus salivarius subsp. Thermophilus, 4 Enterococcus durans, 5 streptococcus equinus.

Identified *Leuc. mesenteroides subsp. Cremoris*were hetero fermentative, capable of producing CO_2 from glucose and fermenting lactose (Table 3). According toStiles and Holzapfel [34], Hemme and Foucaud-Scheunemann [49], Ogier, Casalta [50] the genus of Leuc. are classified as opportunistic pathogens, they are GRAS for use in food fermentations. Leuc. spp. are used in industrial dairy starters.

Pediococcusdamnosus was homo fermentative and producing acid from glucose, galactose fructose (Table 3). Even though pediococci grow inadequately in milk due to their irregular utilisation of lactose, according to Stiles and Holzapfel [34]Carbohydrate utilisation patterns in this genus differ between species, but most pediococci produce lactic acid from glucose.

Table 3 : Identification and carbohydrate fermentation by LAB isolates (API 50 CHL)

	Isolates of LAB						
Carbohydrates	6	7	8	9	10	11	12
Control (No sugar)	-	-	-	-	-	-	-
Glycerol	-	-	-	-	-	-	-
Erythritol	-	-	-	-	-	-	-
D - arabinose	-	-	-	-	-	-	-
L - arabinose	-	-	-	-	-	+	-
D - ribose	-	-	-	+	-	+	-
D - xylose	-	-	-	-	-	+	-
L - xylose	-	-	-	-	-	-	-
D - adonitol	-	-	-	-	-	-	-
Xyloside	-	-	-	-	-	-	-
D - galactose	+	+	-	+	-	+	-
D - glucose	+	+	+	+	+	+	+
D - fructose	-	+	+	+	+	+	+
D - mannose	-	+	-	+	+	-	+
L - sorbose	-	-	-	-	-	-	-
L - rhamnose	-	-	-	-	-	-	-
Dulcitol	-	-	-	-	-	-	-
Inositol	-	-	-	-	-	-	-
Mannitol	-	-	-	+	-	-	-
Sorbitol	-	-	-	+	-	-	-
D - mannoside	-	-	-	-	-	-	-
D - glucoside	-	-	-	-	-	-	-
Glucosamine	+	+	-	+	+	-	-
Amygdalin	-	-	-	+	-	-	-
Arbutin	-	-	-	+	-	-	-
Esculin	-	+	-	+	-	-	-
Salicin	-	-	-	+	-	-	-
Cellobiose	-	-	-	+	-	-	-
Maltose	-	-	-	+	-	+	+
Lactose	+	-	+	+	+	+	+
Melibiose	-	-	-	+	-	+	-
Sucrose	-	-	-	+	+	+	-
Trehalose	-	+	-	+	+	-	+
Inulin	-	-	-	-	-	-	-
Melizitose	-	-	-	+	-	-	-
D - raffinose	-	-	-	-	-	+	-
Starch	-	-	-	-	-	-	-
Glycogen	-	-	-	-	-	-	-
Xylitol	-	-	-	-	-	-	-
Gentiobiose	-	-	-	+	-	-	-
D-Turanose	-	-	-	-	-	-	-
L - Lyxosis	-	-	-	-	-	-	-
D -Tagatosis	-	-	-	-	-	-	-
D - Fucose	-	-	-	-	-	-	-
L - Fucose	-	-	-	-	-	-	-
D - Arabitol	-	-	-	-	-	-	-
L - Arabitol	-	-	-	-	-	-	-
Gluconate	-	-	-	+	-	+	-
2 - Gluconate	-	-	-	-	-	-	-
5 - Gluconate	-	-	-	-	-	-	-

+: Positive reaction: Negative reaction;

6 Leuconostocmesenteroidessubsp. Cremoris, 7 Pediococcusdamnosus, 8 Lactobacillus delbrueckiisubsp. Bulgaricus, 9 Lactobacillus plantarum, 10 Lactobacillus delbrueckiisubsp. Lactis, 11 Lactobacillus brevis, 12 Lactobacillus delbrueckiisubsp.delbrueckii.

The identification profiles of the isolated lactobacilli are shown in Table 3 They were identified as *Lactobacillus delbrueckii subsp. Bulgaricus*, *Lactobacillus delbrueckii subsp. Lactis*, *Lactobacillus delbrueckii subsp. Delbrueckii*, all of these species are homo fermentative, fermenting lactose, glucose and fructose. The other species were identified as *Lactobacillus plantarum*which is homo fermentative, however, *Lactobacillus brevis* is hetero fermentative, both species have the ability to ferment glucose, fructose, galactose and lactose.

Our results show 12 strains of lactic acid bacteria isolated form row cow's milk who can play an important role in the development of organoleptic characteristics which is in accordance with other reported studies [14, 28, 31, 51].

Conclusion

Lactic acid bacteria isolated from raw cow milk represented by Streptococcus, Lactococcus, Lactobacillus, and Enterococcus have been a good result (heamolyses, gram, catalase, sensitivity to salt…). The most dominant is Lactobacillus (41.66%), Streptococcus and Lactococcus (16.66%) followed by Pediococcus,Leuconostocs and Enterococcus (8.33%).

The main identified species are: *Streptococcus salivarius subsp. Thermophilus, Streptococcus equinus, Lactobacillus plantarum*, *Lactobacillus delbrueckii subsp. Lactis*, *Lactobacillus brevis, Lactobacillus delbrueckiisubsp.delbrueckii, Lactobacillus delbrueckiisubsp, Bulgaricus*,. *Lactococcus lactissubspcremoris, Lactococcus lactissubsp. Lactis, Leuconostocmesenteroidessubsp. Cremoris, Pediococcusdamnosus. and Enterococcus durans.*

From the results obtained, bacterial species isolated from raw cow's milk can be used as starter cultures for the manufacture of fermented dairy products, which initiate a rapid acidification of the raw material. These can contribute to microbial safety or offer one or more organoleptic, technological, nutritional or health benefits.

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